

## SHORT COMMUNICATION

# A critical evaluation of the tumor-targeting properties of bispecific antibodies based on quantitative biodistribution data

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**Bispecific and bifunctional antibodies are attracting considerable interest as innovative anti-cancer therapeutics, but their ability to selectively localize at the tumor site has rarely been studied by quantitative biodistribution studies in immunocompetent animal models or in patients. Here, we describe the production of a novel bifunctional antibody, consisting of the F8 antibody (specific to the alternatively spliced EDA domain of fibronectin) fused to the extracellular portion of CD86 (co-stimulatory molecule B7.2). However, the fusion molecule was unable to target tumors *in vivo*. These data suggest that bispecific antibodies do not always localize on tumors and should therefore be characterized by imaging or biodistribution studies.**

**Keywords:** antibody F8/bispecific antibody/co-stimulatory molecule B7.2/oncofetal fibronectin/vascular targeting

## Introduction

Bispecific and bifunctional antibodies represent a novel class of anti-cancer biopharmaceuticals, which are developed with the aim to activate an immune response at the tumor site, while sparing normal organs (Hammond *et al.*, 2007; Nagorsen and Baeuerle, 2011; Pasche and Neri, 2012). Several bispecific antibody formats have been considered and a number of products have been moved to clinical trials (Kontermann, 2005). Promising therapeutic results have recently been reported for clinical trials in patients with advanced hematological malignancies (Topp *et al.*, 2011). Similarly, the field of ‘immunocytokines’ (i.e. antibody-cytokine fusion proteins) has rapidly advanced. Several immunocytokines have been tested in preclinical models of cancer and nine products have been moved to Phase I/II clinical trials (Kontermann, 2012; Pasche and Neri, 2012), with promising results for the treatment of metastatic melanoma (Eigentler *et al.*, 2011).

In our lab, we have extensively studied many immunocytokines in quantitative biodistribution studies in tumor-bearing

mice, using radioiodinated protein preparations and antibodies of proven tumor-targeting performance. While fusion proteins based on certain cytokines or growth factors, such as interleukins (IL, e.g., IL2, IL12, IL15), tumor necrosis factor (TNF), vascular endothelial growth factor (VEGF, e.g., VEGF-120) and granulocyte-macrophage colony-stimulating factor (GM-CSF) retained the excellent tumor-targeting properties of the parental antibody, other immunocytokines or bifunctional proteins (e.g. those based on interferon (IFN)- $\gamma$ , trans-acting activator of transcription (TAT) peptides, calmodulin or dual cytokine fusions) completely abrogated tumor targeting at pharmacologically relevant concentrations, while being fully immunoreactive *in vitro* and displaying good pharmaceutical quality in biochemical assays (e.g. sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and gel-filtration analysis) (Pasche and Neri, 2012).

To our knowledge, there is only one report in the literature describing biodistribution studies of a bispecific antibody (Stork *et al.*, 2009). Unfortunately, the study provides little information about the tumor-targeting properties of the product, as it was performed in immunocompromised mice. In principle, a bispecific antibody capable of avid binding to circulating leukocytes (e.g. T-cells) should have problems extravasating and reaching tumor cells outside the vascular compartment.

Performing biodistribution studies with bispecific antibodies is technically challenging, as it is difficult to demonstrate that both antibody moieties display full immunoreactivity. Indeed, in certain bispecific antibody formats, variable domains may swap pairing, thus leading to a set of molecules with reduced immunoreactivity (Little and Kipriyanov, 2007; Molhoj *et al.*, 2007). Furthermore, it is difficult to measure the immunoreactivity towards antigens, which are not available as pure protein, and which cannot be used in affinity chromatography procedures (e.g. CD3 components).

In order to generate and characterize the properties of a bifunctional antibody molecule, with avid binding to T-cells and to a validated tumor-associated antigen, we fused the F8 antibody (specific to the alternatively spliced EDA domain of fibronectin, a marker of tumor angiogenesis; Rybak *et al.*, 2007; Villa *et al.*, 2008) with the extracellular portion of murine CD86 (B7.2; Sharpe and Freeman, 2002). B7.2 binds to CD28 on T-cells with a dissociation constant  $K_d = 20 \mu\text{M}$  (Collins *et al.*, 2002). When avidity effects come into play, due to the multivalent display of CD28 and CD86 on the cell membrane, the functional affinity of the interaction increases. Similarly, anti-CD3 antibody (Dreier *et al.*, 2002) moieties with  $K_d$  values in the  $0.1 \mu\text{M}$  range have extensively been used for the preparation of bispecific antibodies (Kipriyanov *et al.*, 1999; Brischwein *et al.*, 2006).

The binding properties of B7.2-based bispecific fusion proteins can be readily tested by affinity chromatography, using the tumor-associated antigen or murine CTLA-4 immobilized on resin (Eagar et al., 2002).

In our study, we observed that the F8 antibody efficiently targets tumors *in vivo*, but completely loses its tumor-targeting ability when fused to murine B7.2. These data suggest that the analysis of the tumor-targeting properties of bispecific and bifunctional antibodies by quantitative biodistribution studies should represent an essential activity in product development strategies, since therapeutic performance correlates with the ability of the biopharmaceutical to localize at the site of disease (Carnemolla et al., 2002; Halin et al., 2002b; Wagner et al., 2008).

## Methods

### Cell lines and mouse tumor model

HEK 293 cells (CRL-1573, ATCC) were cultured in Freestyle 293 expression medium (Gibco) containing 0.1% Pluronic F68 (Sigma) in a shaker incubator at 37°C and 5% CO<sub>2</sub>. The murine teratocarcinoma F9 cell line (CRL-1720, ATCC) was cultured as described before (Pasche et al., 2011). Female 129SvPas and Balb/c nude mice were obtained from Charles River (Germany).

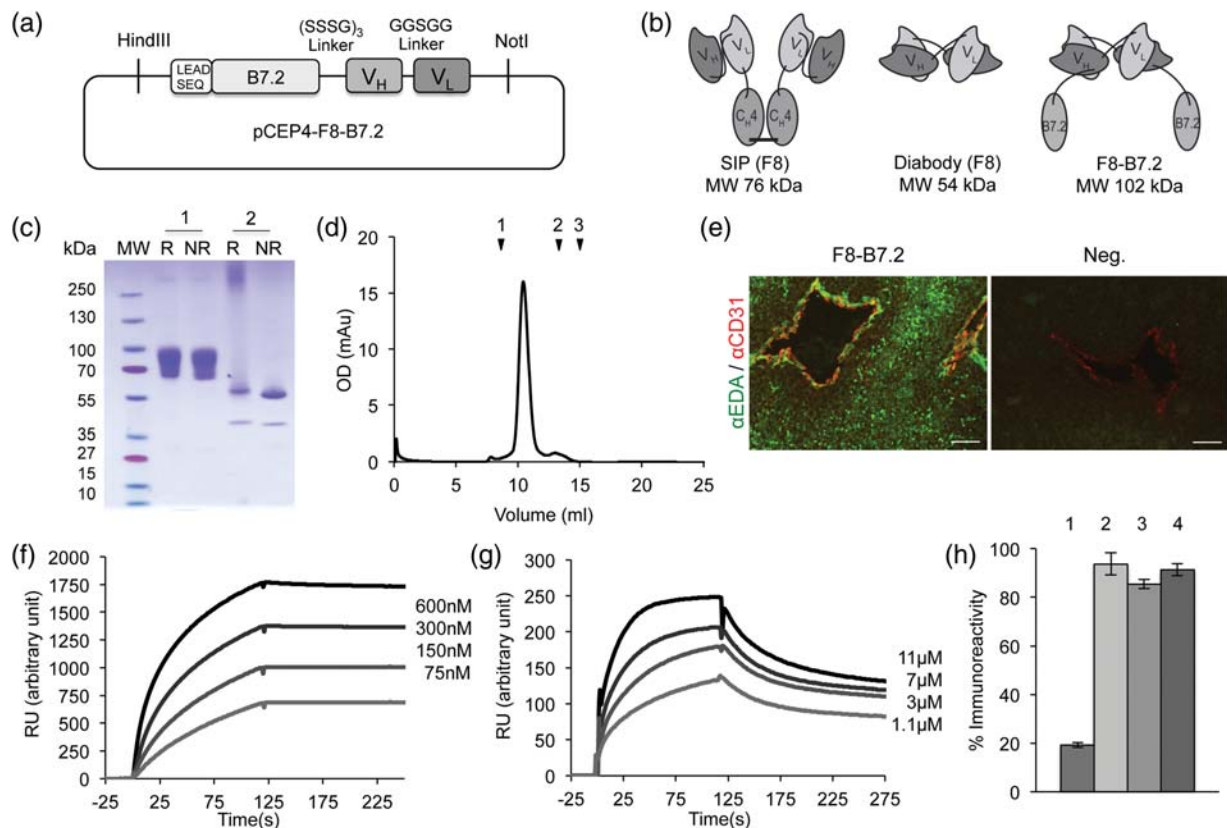
### Cloning and protein expression

The gene structure for SIP(F8) and for the F8 diabody (Villa et al., 2008) has been previously described. F8-B7.2 contains the extracellular domain of murine B7.2 (gene from Source BioScience) sequentially fused to the diabody (Pasche et al., 2011).

The fusion protein was expressed using transient gene expression in HEK-293 cells (Backliwal et al., 2008) and purified by protein A chromatography. The purified protein was analyzed by SDS-PAGE before and after deglycosylation with PNGase F (NEB), size exclusion chromatography (Superdex200 10/300GL, GE Healthcare), BIAcore on a EDA antigen-coated sensor chip and immunofluorescence staining of F9 tumor sections as previously described (Pasche et al., 2011).

### Biodistribution studies

The *in vivo* targeting performance was assessed by quantitative biodistribution studies as described before (Pasche et al., 2011) and 15 µg of radioiodinated F8-B7.2 was injected into the tail vein of tumor-bearing mice. Mice were sacrificed at different time points after injection. Experiments were performed under a project license granted by the Veterinaeramt des Kantons Zurich (169/2008).



**Fig. 1.** Cloning, expression and characterization of F8-B7.2. (a) Schematic representation of the cloning strategy of F8-B7.2. Lead Seq, leader sequence peptide; V<sub>H</sub>, V<sub>L</sub>, variable heavy and light chain. (b) Domain assembly of Sip (F8), diabody (F8) and F8-B7.2. (c) SDS-PAGE analysis of purified F8-B7.2. MW, molecular marker; 1, F8-B7.2; 2, deglycosylated F8-B7.2; NR, R, non-reducing and reducing conditions. (d) Gel filtration analysis of affinity-purified F8-B7.2. 1, Thyroglobulin 669 kDa; 2, BSA 67 kDa; 3, β-lactoglobulin 35 kDa. (e) Immunofluorescence analysis of murine F9 tumors. Tumor sections were stained with a biotinylated F8-B7.2 (green, Alexa488) and an anti-CD31 antibody (red, Alexa594). Magnification ×20, scale bars = 100 µm. (f) BIAcore analysis of purified F8-B7.2 on an EDA-coated sensor chip. (g) BIAcore analysis of F8-B7.2 on a CTLA-4-Fc-coated sensor chip. (h) Immunoreactivity of <sup>125</sup>I-SIP (F8) and <sup>125</sup>I-F8-B7.2 on EDA-resin and on CTLA-4-Fc-resin: 1, <sup>125</sup>I-SIP (F8) on CTLA-4-Fc-resin (*n* = 3); 2, <sup>125</sup>I-SIP (F8) on EDA-resin (*n* = 3); 3, <sup>125</sup>I-F8-B7.2 on CTLA-4-Fc resin (*n* = 3); 4, <sup>125</sup>I-F8-B7.2 on EDA-resin (*n* = 3).

### Immunoreactivity testing

CNBr-activated sepharose (GE Healthcare) was coupled to EDA or to murine CTLA-4-Fc. The retention of radioiodinated F8-B7.2 on resin was analyzed by affinity chromatography, as described (Pasche *et al.*, 2011).

### Results and discussion

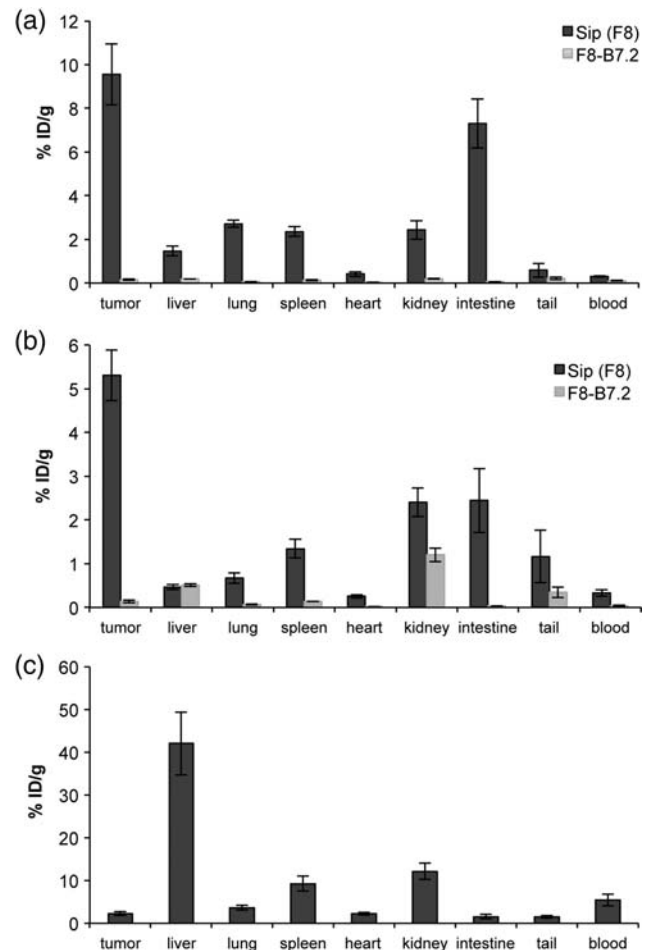
The fusion protein F8-B7.2 was expressed in non-covalent homodimeric 'diabody' format (Fig. 1a and b), leading to fully immunoreactive products with bivalent (i.e. avid) tumor binding and long residence time on neoplastic lesions *in vivo* (Pasche and Neri, 2012). The corresponding F8 antibody, in diabody format and in Small Immune Protein (SIP) format (Fig. 1b), has been extensively studied in biodistribution experiments (Villa *et al.*, 2008). F8-B7.2 was purified to homogeneity by affinity chromatography and was well behaved in SDS-PAGE (Fig. 1c and d). The fusion protein displayed a high degree of N-linked glycosylation, as evidenced by SDS-PAGE analysis before and after N-glycanase treatment. F8-B7.2 stained neo-vascular structures in F9 murine tumors (Fig. 1e) and displayed full retention of immunoreactivity in BIAcore (Fig. 1f and g) and in affinity chromatography procedures with immobilized EDA domain of fibronectin or immobilized murine CTLA-4-Fc fusion (Fig. 1h).

When tested in biodistribution studies in immunocompetent (Fig. 2a) and in nude mice (Fig. 2b) carrying subcutaneously grafted F9 tumors, a radioiodinated preparation of F8-B7.2 failed to preferentially localize on the tumor 24 h after intravenous (i.v.) injection, while the F8 antibody in SIP format (Fig. 2a and b) and in diabody format (Villa *et al.*, 2008) efficiently targeted the neoplastic lesions. Biodistributions at earlier time points (e.g. 15 min after i.v. injection; Fig. 2c) revealed that the majority of the protein had already been excreted via the hepatobiliary route, while never reaching the cognate antigen *in vivo*, which is localized on the abluminal side of tumor blood vessels (Rybak *et al.*, 2007; Villa *et al.*, 2008).

The selective localization of antibody-fusion proteins to the site of disease *in vivo* is strictly dependent on the payloads that are attached to the antibody. Our group observed that highly charged payloads (e.g. Calmodulin (Melkko *et al.*, 2002), VEGF-164 (Halin *et al.*, 2002a) and HIV-1 TAT peptides (Niesner *et al.*, 2002)), bulky payloads (Gafner *et al.*, 2006) and payloads prone to receptor trapping when administered at low doses (e.g. IFN $\gamma$ ; Ebbinghaus *et al.*, 2005) can completely abrogate tumor targeting.

Our data suggest that it is not obvious that a bifunctional protein with avid binding to circulating leukocytes would efficiently extravasate and reach a tumor-associated antigen *in vivo*, even if the parental antibody displays good tumor-targeting properties. In the case of F8-B7.2, the tumor-targeting process does not take place even in immunocompromised mice and is prevented by the rapid clearance from blood through the liver. Due to its heavily glycosylated nature, the fusion protein is subject to binding by carbohydrate-recognizing receptors on hepatocytes and the resulting efficient and fast blood clearance rate prohibits the targeting to the site of disease.

Some bispecific and bifunctional antibodies have demonstrated a dramatic therapeutic activity in certain clinical trials and it is possible that even small amounts of therapeutic



**Fig. 2.** Biodistribution study of radioiodinated F8-B7.2. Mice bearing subcutaneous F9 teratocarcinoma were injected i.v. with 15  $\mu$ g radiolabeled protein. (a) Immunocompetent 129SvPas mice injected with  $^{125}$ I-SIP (F8) (black bar,  $n = 5$ ) or with  $^{125}$ I-F8-B7.2 (gray bar,  $n = 4$ ). Mice were sacrificed after 24 h. (b) Nude mice injected with  $^{125}$ I-SIP (F8) (black bar,  $n = 5$ ) or with  $^{125}$ I-F8-B7.2 (gray bar,  $n = 4$ ). Mice were sacrificed after 24 h. (c) Nude mice injected with  $^{125}$ I-F8-B7.2 ( $n = 4$ ). Mice were sacrificed after 15 min. Organs were excised and radioactivity counted, expressing results as percent of injected dose per gram of tissue (%ID/g  $\pm$  SE).

protein, delivered at the site of disease, may mediate a potent anti-tumoral immune response. However, there is ample evidence that efficient tumor localization of bifunctional antibodies correlates with therapeutic performance and a quantitative biodistribution study of targeting should represent an essential requirement for future product development in this field.

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**Contribution:** T.H. conceived the experiments, produced the protein and performed biodistribution studies and wrote the manuscript; S.W. developed TGE methodologies for protein expression, helped in biodistribution studies and corrected the manuscript; D.N. conceived the experiments, supervised the experimental work and wrote the manuscript.

**Conflict of interest:** Dario Neri is a co-founder and shareholder of Philogen ([www.philogen.com](http://www.philogen.com)), the company which owns the F8 antibody.



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